Mouse chronic social stress increases blood and brain kynurenine pathway activity and fear behaviour: both effects are reversed by inhibition of indoleamine 2,3-dioxygenase

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Abstract: Psychosocial stress is a major risk factor for mood and anxiety disorders, in which excessive reactivity to aversive events/stimuli is a major psychopathology. In terms of pathophysiology, immune-inflammation is an important candidate, including high blood and brain levels of metabolites belonging to the kynurenine pathway. Animal models are needed to study causality between psychosocial stress, immune-inflammation and hyper-reactivity to aversive stimuli. The present mouse study investigated effects of psychosocial stress as chronic social defeat (CSD) versus control-handling (CON) on: Pavlovian tone-shock fear conditioning, activation of the kynurenine pathway, and efficacy of a specific inhibitor (IDOInh) of the tryptophan-kynurenine catabolizing enzyme indoleamine 2,3-dioxygenase (IDO1), in reversing CSD effects on the kynurenine pathway and fear. CSD led to excessive fear learning and memory, whilst repeated oral escitalopram (antidepressant and anxiolytic) reversed excessive fear memory, indicating predictive validity of the model. CSD led to higher blood levels of TNF- , IFN- , kynurenine (KYN), 3-hydroxykynurenine (3-HK) and kynurenic acid, higher KYN and 3-HK in amygdala and hippocampus. However, CSD was without effect on IDO1 gene or protein expression in spleen, ileum and liver, whilst increasing liver TDO2 gene expression. Nonetheless, oral IDOInh reduced blood and brain levels of KYN and 3-HK in CSD mice to CON levels, and we therefore infer that CSD increases IDO1 activity by increasing its post-translational activation. Furthermore, repeated oral IDOInh reversed excessive fear memory in CSD mice to CON levels. IDOInh reversal of CSD-induced hyper-activity in the kynurenine pathway and fear system contributes significantly to the evidence for a causal pathway between psychosocial stress, immune-inflammation and the excessive fearfulness that is a major psychopathology in stress-related neuropsychiatric disorders.

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Mouse chronic social stress increases blood and brain kynurenine pathway activity and fear behaviour: both effects are reversed by inhibition of indoleamine 2,3-dioxygenase

Running title: Mouse stress, kynurenines and fear behaviour

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ABSTRACT

Psychosocial stress is a major risk factor for mood and anxiety disorders, in which excessive reactivity to aversive events/stimuli is a major psychopathology. In terms of pathophysiology, immune-inflammation is an important candidate, including high blood and brain levels of metabolites belonging to the kynurenine pathway. Animal models are needed to study causality between psychosocial stress, immune-inflammation and hyper-reactivity to aversive stimuli. The present mouse study investigated effects of psychosocial stress as chronic social defeat (CSD) versus control-handling (CON) on: Pavlovian tone-shock fear conditioning, activation of the kynurenine pathway, and efficacy of a specific inhibitor (IDOInh) of the tryptophan-kynurenine catabolizing enzyme indoleamine 2,3-dioxygenase (IDO1), in reversing CSD effects on the kynurenine pathway and fear. CSD led to excessive fear learning and memory, whilst repeated oral escitalopram (antidepressant and anxiolytic) reversed excessive fear memory, indicating predictive validity of the model. CSD led to higher blood levels of TNF-α, IFN-γ, kynurenine (KYN), 3-hydroxykynurenine (3-HK) and kynurenic acid, and higher KYN and 3-HK in amygdala and hippocampus. CSD was without effect on IDO1 gene or protein expression in spleen, ileum and liver, whilst increasing liver TDO2 gene expression. Nonetheless, oral IDOInh reduced blood and brain levels of KYN and 3-HK in CSD mice to CON levels, and we therefore infer that CSD increases IDO1 activity by increasing its post-translational activation. Furthermore, repeated oral IDOInh reversed excessive fear memory in CSD mice to CON levels. IDOInh reversal of CSD-induced hyper-activity in the kynurenine pathway and fear system contributes significantly to the evidence for a causal pathway between psychosocial stress, immune-inflammation and the excessive fearfulness that is a major psychopathology in stress-related neuropsychiatric disorders.

Keywords: psychosocial stress; fear conditioning; inflammation; IDO1; kynurenine pathway; monoamines; depression; anxiety

1. Introduction

Stress-related neuropsychiatric disorders, such as major depressive disorder (MDD) and generalized anxiety disorder (GAD), are among the most significant contributors to global disease burden (Collins et al., 2011). Current treatments are often inadequate (Mojtabai, 2009; Wittchen et al., 2011), in large part due to the related issues that each disorder comprises heterogeneous groups of symptoms (DSM-5, 2013) and their aetio-pathophysiologies are poorly understood. Furthermore, these disorders often present co-morbidly, either with each other or together with other diseases including autoimmune disorders (Bruce, 2008; Siegert and Abernethy, 2004). To help address this situation, specific emotional and cognitive dysfunctions, or research domain criteria (RDoC), have become an important focus in translational psychiatric research, aimed at improved understanding of the aetio-pathophysiology of, and development of novel pharmacotherapies for specific psychopathologies (Cuthbert and Insel, 2013; Insel et al., 2010).

Negative valence is a major proposed domain (Cuthbert and Insel, 2013), and includes the excessive fearfulness that is common in MDD and GAD (Disner et al., 2011; Eshel and Roiser, 2010). Importantly, these domains can be studied in humans and other species. A paradigm for the study of aversive-stimulus processing in humans and rodents is Pavlovian fear conditioning: repeated pairing of an initially neutral conditioned stimulus (CS) with an aversive unconditioned stimulus (US) results in the CS acquiring negative valence and eliciting emotional responses (Rescorla, 1988). In human and rodent, the US is usually mild-moderate electroshock (Fullana et al., 2015; Phelps and LeDoux, 2005), and visual and auditory CS are typically used in human and rodent, respectively (Fullana et al., 2015; Phelps and LeDoux, 2005). In humans, CS responsiveness is measured using skin conductance and subjective report (Fullana et al., 2015), and in rodents the defensive behaviour of complete

inactivity, or “freezing”, is used (for review: (Blanchard et al., 2001; Maren and Quirk, 2004; Phelps and LeDoux, 2005)). Rodent fear conditioned freezing provides a model system for investigating the neurobiology of negative valence processing, including CS-US learning, consolidation and recall of CS-US memory, and activation of conditioned freezing response (LeDoux, 2000; Rescorla, 1988; Wolff et al., 2014). The amygdala, specifically the basolateral complex and central nucleus, is essential for each of these stages (Duvarci and Pare, 2014; Kim and Jung, 2006; Maren and Quirk, 2004). In human, CS-US fear conditioning has been demonstrated to be increased in both MDD (Nissen et al., 2010) and GAD (Greenberg et al., 2013; Lissek et al., 2015).

In terms of aetio-pathophysiology of these psychiatric disorders, stress-induced activation of immune-inflammation is an important candidate (for review: (Dantzer et al., 2008; Miller et al., 2009)). Acute psychosocial stress increases blood levels of chemokines and cytokines (Bierhaus et al., 2003), and chronic psychosocial stress leads to sensitization of stress inflammatory responses (Rohleder, 2014). MDD is associated with increased blood levels of the pro-inflammatory cytokines tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) (Dowlati et al., 2010). MDD patients exhibit up-regulation of *TNF* and other inflammation genes in peripheral blood mononuclear cells; the expression levels of these genes correlate with amygdala reactivity to fearful faces (Savitz et al., 2013). One candidate stress-activated inflammation pathway, including for induction of hyper-fearfulness, is the kynurenine pathway: increased TNF-α, IL-6 and interferon-γ (IFN-γ) increase expression of indoleamine 2,3-dioxygenase (IDO1) and tryptophan 2,3-dioxygenase (TDO2), enzymes expressed by immune cells and other cell types in various tissues, that promote tryptophan (TRP) catabolism to kynurenine (KYN) in periphery and brain (Dai and Zhu, 2010; Gibney et al., 2014; Robinson et al., 2003; Vecsei et al., 2013; Werner-Felmayer et

al., 1989). KYN is further metabolized to, among others, 3-hydroxykynurenine (3-HK), quinolinic acid (QUIN) and kynurenic acid (KYNA), catabolites that can contribute to oxidative stress, excitotoxicity, and neuroprotection (for review: (Chiarugi et al., 2001; Haroon et al., 2012; Schwarcz et al., 2012). There is growing evidence for kynurenine-pathway hyperfunction in stress-related disorders, particularly for MDD (Bay-Richter et al., 2015; Kim et al., 2012; Reus et al., 2015; Savitz et al., 2015; Steiner et al., 2011; Sublette et al., 2011) (for review: (Maes et al., 2011)).

Valid animal models are essential for the investigation of causal inter-relationships between psychosocial stress, activation of immune-inflammation, and induction of changes in brain and behaviour. Social disruption stress induces increased blood levels of pro-inflammatory cytokines and inflammatory monocytes, splenomegaly, increased monocyte trafficking to the brain, microglia activation in amygdala, hippocampus and prefrontal cortex, and hyper-anxiety (Hanke et al., 2012; Wohleb et al., 2011; Wohleb et al., 2013). Chronic exposure to social stress by an aggressive strain (chronic social defeat, CSD) induces increased blood levels of pro-inflammatory cytokines (TNF-α, IL-6) and splenomegaly (Azzinnari et al., 2014; Savignac et al., 2011), and CSD mice exhibit increased social avoidance ((Krishnan et al., 2007), see above), decreased active avoidance of footshock US (Azzinnari et al., 2014), and excessive CS fear expression (Yu et al., 2011).

The aims of the present study were to: (1) Establish a mouse model comprising CSD-induced excessive CS-US fear learning and memory and to validate it with the antidepressant and anxiolytic escitalopram. (2) Investigate whether CSD induces activation of the kynurenine pathway in the circulation and in brain regions, most notably the amygdala, relevant to fear learning and memory. (3) Investigate whether a specific inhibitor of IDO1 reverses kynurenine pathway activation. (4) Investigate whether activation of the

kynurenine pathway contributes to excessive fear in CSD mice using the IDO1 inhibitor. Should IDO1 inhibition reverse both kynurenine pathway hyperactivity and excessive fear in CSD mice, this would constitute significant translational evidence for a causal pathway between psychosocial stress, a specific immune-inflammation pathway, and excessive fearfulness in psychiatric disorders.

2. Materials and Methods

2.1. Animals

The study was conducted with C57BL/6J mice bred in-house (Zurich). Male offspring were weaned aged 3 weeks and caged in groups of 2-3 littermates; a total of 160 mice born to 60 breeding pairs were studied. Mice were aged 12 weeks and weighed 27-31 g at study onset. Male CD-1 mice (N=75, Janvier Labs, Saint-Berthevin, France) were aged 8 months, were ex-breeders, and caged singly. Mice were maintained on a reversed 12:12 h light-dark cycle (lights off 07:00-19:00 h) in an individually-ventilated caging system at 20-22°C and 50-60 % humidity. Cages were type 2L (20 x 35 x 13 cm) and contained wood chips, a sleeping igloo and tissue bedding. Complete-pellet diet (Provimi, Kliba AG, Kaiseraugst, Switzerland) and water were available continuously. The study was conducted under a permit (170/2012) issued by the Veterinary Office, Zurich, Switzerland. All efforts were made to minimize the number of mice studied and any unnecessary stress to those mice that were studied.

2.2. Chronic social defeat

Chronic social defeat (CSD) was conducted using a refined protocol as described elsewhere (Azzinnari et al., 2014). Briefly, a C57BL/6 (CSD) mouse was housed singly in the home cage of a CD-1 mouse separated by a transparent, perforated divider. The CSD mouse was placed

with the CD-1 mouse for either a cumulative total of 60 sec physical attack or 10 min maximum. To avoid bite wounds, essential for the study of psychosocial stress-induced immune-inflammation, the lower incisors of CD-1 mice were trimmed every third day across CSD (Azzinnari et al., 2014). Each day for 15 days, the CSD x CD-1 mouse pairings were rotated so that CSD mice were confronted daily with a novel CD-1 mouse. On days 16-20 depending on experiment (see below), CSD mice remained adjacent to the same CD-1 mouse without any further attacks. Control (CON) mice remained in littermate pairs and were handled and weighed daily. A validation experiment comparing CON mice pairs maintained together or separated by a divider, demonstrated the absence of a caging effect on fear conditioning.

2.3. Escitalopram

The selective serotonin reuptake inhibitor (SSRI) escitalopram oxalate (ESC) was supplied by Lundbeck (H. Lundbeck A/S, Valby, Denmark). A solution of 10 mg/kg/8 ml (expressed as salt) was prepared in Natrosol (0.5 %) as vehicle and protected from light. Escitalopram was administered using intragastric gavage (per os, p.o.) at 16:00 h on day 15 and at 1 h prior to behavioural testing on days 16-18.

2.4. Indoleamine 2,3-dioxygenase inhibitor

A small-molecule, potent (both human and murine) and selective inhibitor of indoleamine 2,3-dioxygenase 1 (IDO1) (hereafter IDOInh) (see (Liu et al., 2010; Yue et al., 2009) and (Cathomas et al., 2015)) was used. As reported elsewhere, in cellular assays of potency to block tryptophan-to-KYN conversion, the *in vitro* 50 % inhibitory concentration (IC_{50}) on murine IDO1 was 50 nM (Liu et al., 2010). For murine IDO2 IC_{50} was >5000 nM and for

human TDO2 it was >10000 nM; therefore, *in vitro* selectivity of IDOInh for IDO1 relative to IDO2 was >100 and relative to TDO2 was >200 (Liu et al., 2010). In an in-house cellular assay, IC_{50} on murine IDO1 was 390 nM. For pharmacokinetic analysis (Fig. 4A), IDOInh was formulated as a suspension in 15 % hydroxypropyl-β-cyclodextrin (HP-β-CD) in 0.5 % Natrosol and administered at a dose of 200 mg/kg p.o.. The time required to reach peak plasma exposure was 1.3 h, and the mean residence time (average time that an IDOInh molecule remains in the blood) was 4.6 h (N=3 mice; Fig. 4A). Two days later the same mice received 200 mg/kg p.o. and total plasma exposure at 2 h was 20.7 µM; given that plasma protein binding was 96.7 %, this would yield plasma protein-unbound exposure of about 680 nM, 1.8x IC_{50} of IDO1. The *in vivo* efflux, calculated as muscle/brain ratio, was 14; this low brain value was consistent with *in vitro* transporter experiments showing that IDOInh is a substrate for the transporters P-gp (P-glycoprotein) and BCRP (breast cancer resistance protein) expressed in the blood-brain barrier. Accordingly, cerebrospinal fluid exposure at 2 h was 60 nM, 0.2x IC_{50} of IDO1. After induction of high plasma KYN by lipopolysaccharide (LPS, 1 mg/kg i.p.), which up-regulates *Ido1* expression (O'Connor et al., 2009b), at 24 h previously, 200 mg/kg p.o. IDOInh led to complete reversal of the increase in plasma KYN at 1 h post-administration; the 50 % effective dose (ED_{50}) was 10-30 mg/kg p.o.. The IDOInh dose selected for experiments was 200 mg/kg/8 ml, using 0.5 % Natrosol in water as vehicle. IDOInh or vehicle-only were administered using intragastric gavage at 16:00 h on day 15, and at 2 h prior to behavioural testing or blood and brain collection on days 16-18.

2.5. Experimental design

Four complementary experiments were conducted, each with a different, naive cohort of mice (Figure 1). In week 1 of each experiment, mice were handled and weighed on five

consecutive days. Experiment A investigated the effects of CSD or CON X p.o. escitalopram or vehicle at days 15-18 (CON-VEH N=12, CON-ESC N=10, CSD-VEH N=15, CSD-ESC N=15), on freezing behaviour in a neutral arena (day 16) and during fear conditioning (day 17) and fear expression (day 18). At day 20, to obtain a first indication of CSD effects on the kynurenine pathway - albeit confounded by exposure to the fear conditioning paradigm - from a sample of CON-VEH (N=8) and CSD-VEH (N=7) mice, terminal blood and brain samples were collected for determination of pro-inflammatory cytokines and kynurenines. Experiment B investigated the effects of CSD or CON X single p.o. IDOInh or VEH at day 16 (CON-VEH N=8, CON-IDOInh N=8, CSD-VEH N=8, CSD-IDOInh N=8) on plasma and brain levels of kynurenines. Experiment C investigated the effects of CSD (CON N=10, CSD N=10) on gene expression of *Ido1*, *Ido2* and *Tdo2* in spleen, ileum and liver, and of IDO1 protein expression in ileum and liver. Spleen (mononuclear cells) and ileum are tissues with high *Ido1*/IDO1 expression (Dai and Zhu, 2010) and *Tdo2* expression is high in liver (Ren and Correia, 2000). Experiment D investigated the effects of CSD or CON X p.o. IDOInh or VEH at days 15-18 (CON-VEH N=12, CON-IDOInh N=14, CSD-VEH N=12, CSD-IDOInh N=18), on freezing in a neutral arena (day 16) and during fear conditioning (day 17) and fear expression (day 18).

(FIGURE 1 ABOUT HERE)

2.6. Pavlovian fear learning and memory

Behavioural testing (Expt A, D) was conducted under dim lighting in a room adjacent to the mouse holding room, between 09:00-12:00 h (early dark phase), using a Multi-Conditioning System in which activity was monitored continuously using an infrared movement-detection system (TSE Systems GmbH, Bad Homburg, Germany) (Cathomas et al., 2015; Pryce et al., 2012). The main paradigm was Pavlovian fear conditioning, comprising the two stages of

tone CS-footshock US fear conditioning and CS fear expression; these two stages are inter-dependent in that what is learned during the conditioning stage determines what can be consolidated and recalled during the expression stage. Freezing was the readout for conditioning and expression, defined as no detectable movement for at least 2 sec; freezing was measured as percent time spent in this state. Locomotor activity was also measured continuously.

Neutral arena tests. The mouse was placed on the grid floor in an arena (context) for 15 min, and activity and % time freezing were recorded continuously. Two such tests were conducted in each of Experiments A and D: a first test was conducted prior to CSD to determine baseline % time freezing levels, which were then used to counterbalance allocation to CSD and CON groups, and a second test at day 16 assessed CSD and drug effects on freezing and activity levels.

CS-US fear conditioning. On day 17, the mouse was placed on the grid floor in the same arena and, following 5-min adaptation, exposed to 6 trials of a discrete, neutral tone of 5 kHz and 85 dB (conditioned stimulus, CS) presented via a loudspeaker for 20 sec with the final 2 sec contiguous with a 2 sec x 0.15 mA (i.e. mild) footshock (unconditioned stimulus, US). The inter-trial interval (ITI) was 120 sec. Percent time freezing was measured during the six CSs and five ITIs; for analysis, these trials were grouped into CS blocks 1-2, 3-4, 5-6 and ITIs 1, 2-3, 4-5.

Fear expression. On day 18, the mouse was placed on the grid floor in the same arena and, following 5-min adaptation, exposed to nine trials of the tone CS for 30 sec and with an ITI of 90 sec. Percent time freezing was measured during the nine CSs and eight ITIs; for analysis, trials were grouped into CS blocks 1-3, 4-6, 7-9 and ITIs 1-2, 3-5, 6-8.

2.7. Collection of brain, blood, spleen, ileum and liver

Mice were decapitated and trunk blood (Expt A, B) was collected in EDTA-coated tubes (Microvette 500 K3E, Sarstedt) and placed on ice. The brain (Expt A, B) was removed from the skull, rinsed in ice-cold saline, frozen on dry ice and stored at -80°C. Bloods were centrifuged at 3000 rpm and 4°C for 15 min, plasma aliquots were transferred to cryotubes (Protein LoBind, Eppendorf) and stored at -80°C. The spleen (Expt A, C) was removed, cleaned of fat and connective tissue, and weighed or placed in PBS for determination of splenocyte expression of genes for tryptophan-catabolizing enzymes *Ido1*, *Ido2* and *Tdo2*. Pieces of ileum and liver (Expt C) were dissected out and immediately frozen for determination of *Ido1*, *Ido2* and *Tdo2* mRNA and IDO1 protein.

2.8. Brain tissue microdissection

In Expt A and B, frozen brains were placed in a stainless steel mouse brain matrix (Plastics One, model MMCS-1) and cut coronally into 1 mm sections (Azzinnari et al., 2014). Using a brain punch (Ø=1 mm, Stoelting Europe, model 57397) and a mouse brain atlas (Franklin and Paxinos, 2008) the following regions were microdissected: amygdala (1 biopsy/hemisphere) at bregma -1.0 to -2.0 mm, ventral hippocampus (vHIPP, 3 biopsies/hemisphere) at bregma -3.0 to -4.0 mm, medial prefrontal cortex (mPFC, 1 biopsy/hemisphere) at bregma 2.0 to 1.0 mm, dorsal striatum (DS, 2 biopsies/hemisphere) at bregma 0.0 to -1.0 mm, and dorsal raphe nucleus (DRN, 1 medial biopsy) at -4.0 to -5.0 mm. Microdissection was conducted at -18°C and brain biopsies were weighed and stored at -80°C until analysis. Amygdala, vHIPP and mPFC were studied because of their importance in fear conditioning and stress-related psychiatric disorders; DS and DRN were studied based on previous reports that stress increases kynurenines in these regions (Laugeray et al., 2010; Miura et al., 2011). A saline

perfusion step was not included in the brain collection protocol: a pilot study in naive and LPS-treated mice demonstrated comparable tryptophan metabolite levels in brain tissue collected with and without saline perfusion; expression levels of haemoglobin genes (e.g. *Hba-a1*, *Hbb-a2*, also expressed by brain cells (Biagioli et al., 2009)) obtained in brain tissues collected without saline perfusion were markedly lower (<0.02 %) than those measured in blood monocytes (Azzinnari et al., 2014); unpublished data).

2.9. Immunoassay of plasma and brain pro-inflammatory cytokines

In Expt A, determination of immunoreactive TNF-α in plasma and hippocampus was conducted using a flow cytometric particle based assay with a lower limit of quantification (LLOQ) of 0.5 pg/ml (Azzinnari et al., 2014; Marques-Vidal et al., 2011). For hippocampal TNF-α, punches were ultrasonicated in 10x volume of complete Tris lysis buffer, centrifuged, and the supernatant pipetted off for assay. Determination of immunoreactive IFN-γ in plasma and dorsal striatum was performed using an electrochemiluminescence assay (IFN-gamma (mouse) V-Plex Kit, Meso Scale Discovery (MSD), Rockville, MD, USA) with a LLOQ of 0.2 pg/ml. For DS IFN-γ, punches were ultrasonicated in 10x volume of complete MSD Tris lysis buffer, centrifuged, and the supernatant pipetted off for assay. All samples were analysed in duplicate.

2.10. LC-MS/MS for plasma and brain TRP, kynurenines, monoamines and the IDO1 inhibitor

In Expt A and B, frozen plasma sample aliquots and brain biopsies were transferred to Boehringer Ingelheim Biberach, Germany. Plasma and brain levels of tryptophan (TRP), its kynurenine pathway metabolites, and serotonin (5-HT), dopamine (DA) and IDOInh were determined using liquid chromatography-tandem mass spectrometry (LC-MS/MS, (Cathomas

et al., 2015). Plasma samples, calibration standards and quality controls were diluted with mobile phase A (0.2 % formic acid, 0.01 % trifluoroacetic acid, 1 % acetonitrile in water) and a deuterated internal standard mix (d5-tryptophan, d4-kynurenine, d3-quinolinic Acid, d5-kynurenic acid, d4-serotonin). Methanol was added for protein precipitation. Subsequently, samples were centrifuged and supernatant was evaporated to dryness under a gentle stream of nitrogen and reconstituted in mobile phase A. Brain biopsies, calibration standards, quality controls and a deuterated internal standard mix (as for plasma samples, with the addition of d4-dopamine) in 0.1 % formic acid and ice-cold methanol were homogenized by sonication. After centrifugation the supernatant was evaporated to dryness under nitrogen and reconstituted in mobile phase A.

The LC-MS/MS system used to analyze plasma and brain samples consisted of a CTC HTC PAL Autosampler (CTC Analytics AG, Zwingen, Switzerland) and an Agilent 1200 Series liquid chromatography system (Micro Vacuum Degasser, Binary Pump SL, Thermostatted Column Compartment; Agilent Technologies, Waldbronn, Germany), coupled to an API 4000^{TM} triple quadrupol mass spectrometer (AB Sciex, Darmstadt, Germany). Separation of analytes was achieved using a GRACE VisionHT C18 Basic column (3 µm, 100 x 2.1 mm; Grace Davison Discovery Sciences, Lokeren, Belgium) and a linear gradient elution profile. All standards, solvents and reagents used were of highest purity (LC-MS grade where available; Sigma Aldrich; CDN Isotopes, Quebec, Canada; Cambridge Isotope Laboratories Inc, Andover, USA; Buchem BV, Apeldoorn, Netherlands). Mobile phase A consisted of 0.2 % formic acid, 0.01 % trifluoracetic acid, 1 % acetonitrile, in water, and mobile phase B consisted of 0.2 % formic acid, 0.01 % trifluoracetic acid, 1 % water, in acetonitrile. To assess accuracy, quality control samples (from a pool of the study samples) were run at the end of every sample batch. Absolute analyte concentrations in calibration standards, samples and quality control

samples were calculated based on the analyte signal in relation to the signal of the deuterated internal standards; the amount of internal standard added was selected with the aims of being in the range of endogenous analyte concentrations and attaining an adequate signal-to-noise ratio. As demonstrated in a previous study (Cathomas et al., 2015): in plasma samples it was possible to conduct reliable measurement of TRP, KYN, 3-hydroxy-kynurenine (3-HK), kynurenic acid (KYNA), quinolinic acid (QUIN), and 5-HT; in small region-specific brain biopsies (~1 mg of tissue) it was possible to reliably measure TRP, KYN, 3-HK, 5-HT and DA, but KYNA or QUIN were below the LLOQ, which was 0.25 nmol and 5 nmol, respectively. Absolute analyte concentrations were calculated as µmol/l or nmol/l for plasma and µmol/kg or nmol/kg tissue for brain regions.

2.11. Quantitative real-time PCR for tryptophan-catabolizing enzymes

In Expt C, spleens were collected in Dulbecco's phosphate-buffered saline (DPBS 1X) (Gibco), passed through a 70 µm nylon mesh cell strainer (Falcon) into a 6 well-plate. The suspension containing splenic mononuclear cells (SMCs) was transferred to 50 ml tubes, the volume was made up to 50 ml with DPBS, and centrifuged for 5 min at 1800 rpm and RT. The supernatant was discarded, the cells were re-suspended in 1 ml Red Cell Lysis Buffer (Sigma), incubated for 2 min at RT, 49 ml DPBS were added, and cells were centrifuged as above. After discarding the supernatant, cells were re-suspended in 1 ml DPBS, transferred to 1.5 ml tubes (Eppendorf) and centrifuged followed by removal of the supernatant. 1 ml Trizol (Ambion) was added to the cell pellet and samples were stored at -80°C until RNA extraction.

In samples of SMCs, ileum and liver, total RNA was extracted using Trizol (Ambion) according to the manufacturer`s instructions. Cell/tissue lysis was performed using a tissue lyser (Mixer-Mill 300, Qiagen) with stainless steel beads (Ø = 5 mm, Retsch). Isolated RNA

was digested with DNase I (Fermentas), and retrotranscribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression analysis was conducted using SYBR green (Applied Biosystems) and a 7900HT Fast Real-Time PCR System (Applied Biosystems). As the normalization factor (NF), the geometric mean of cycle threshold (Ct) values for the genes *Hprt1* and *Tbp* was used. Ct values of the target genes i.e. *Ido1*, *Ido2* or *Tdo2* were normalized to the NF using the ΔΔCt method (Pfaffl et al., 2002). Relative expression values were log2-transformed for statistical analysis. The list of primers used is given in Table S1.

2.12. Western blot for IDO1

In Expt C, a section of ileum rinsed with 0.9 % NaCl to remove contents (60-80 mg tissue) or a piece of liver (50-60 mg) was suspended in 500 µl ice-cold lysis buffer (50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 % Triton-X-100, 0.1 % SDS, 0.5 % sodium deoxycholate and protease inhibitor (Roche)), sonicated for 2x 10 sec on ice, centrifuged at 12500 rpm at 4°C for 10min, and the supernatant transferred to Protein LoBind tube (Eppendorf). Protein concentration was determined with a BCA Protein Assay Kit (Pierce, Thermo Scientific). Lysates for ileum (20 µg protein) or liver (100 µg protein) were denatured at 95°C for 5 min in 1x Laemmli Buffer, 0.05M dithiothreitol and ddH_2O. Cooled (RT) samples were loaded on a 12.5 % SDS-polyacrylamide gel (Criterion™ TGX™, Bio-Rad) and protein separation was performed at 200 V for 45min. Separated proteins were transferred to nitrocellulose membranes using Trans-Blot Turbo (Transfer System, Bio-Rad) at 1.3 A/25 V for 7 min. Membranes were washed 2x 5min with PBS-T (0.1 % Tween20 in PBS) and were then blocked for 1 h with 5 % nonfat dry milk in PBS-T. After 2x 5 min rinsing with PBS-T, the membranes were incubated overnight at 4°C with rat anti-mouse IDO1 (mIDO-48, BioLegend, 1:1000). The blot was

rinsed 2x 5min with PBS-T, and membranes were incubated 1 h at RT with anti-rat IgG conjugated to horseradish peroxidise (HRP) (AbD Serotec, 1:10'000) and mouse anti-β-actin conjugated to HRP (Sigma-Aldrich, 1:150'000). Membranes were washed 2x with PBS-T, and signal detection was performed with Clarity Western ECL Substrate (Bio-Rad). Band optical densities were measured using ImageJ software (U.S. National Institutes of Health, Bethesda, Maryland, USA) and % densitometry ratio of IDO1 to β-actin was calculated.

2.13. Statistical analysis

Statistical analysis of behavioural measures was conducted using SPSS (version 20, SPSS Inc., Chicago IL, USA): general linear model analysis of variance (ANOVA) with a mixed-design of Stress (CSD, CON) X Drug (Drug, VEH) X Trial-block (ITI or CS trial blocks) was used, with significant effects analyzed using Fisher's least significant difference (LSD) *post hoc* test. Statistical analysis of CSD and IDOInh effects on inflammation factors was conducted using GraphPad Prism (version 6.04), including *t*-test for Stress effects and ANOVA with a Stress x Drug design and LSD *post hoc* testing. Data are expressed as individual scatter plots or mean ± standard error (SE). Statistical significance was set at $p \leq 0.05$.

3. Results

3.1. Physical effects of CSD

In Expt A-D, there was no Stress effect on mean absolute body weight (BW), as measured in 5-day blocks (pre-CSD/CON days 5-1, CSD/CON days 2-6, 7-11, 12-16). For example, in Expt D, CON mice (N=26) weighed 28.8±0.5 g at pre-CON and 29.4±0.5 g at days 12-16, and CSD mice (N=30) weighed 29.7±0.4 g at pre-CSD and 30.3±0.5 g at days 12-16 (Day-block main effect $F_{3,162}=14.45$, $p<0.0005$, Stress main or interaction effect $p \geq 0.20$). However, mean day-

to-day BW delta (ΔBW) was increased in CSD relative to CON mice, and to a similar extent across experiments. In Expt D, for example, there was a Stress x Day block interaction ($F_{3,162}$=7.42, p<0.0005): mean ΔBW was similar in CON and CSD mice at pre-CSD/CON days 5-1 (CON 1.4±0.1 %, CSD 1.3±0.1 %, p=0.73), but higher in CSD than CON mice at days 2-6 (CON 1.0±0.1 %, CSD 1.7±0.1 %, p<0.0005), 7-11 (CON 1.1±0.1 %, CSD 1.5±0.1 %, p<0.006), and 12-16 (CON 1.3±0.1 %, CSD 2.2±0.1 %, p<0.0005). Increased daily ΔBW combined with no effect on absolute BW provides a reproducible marker for CSD efficacy (Azzinnari et al., 2014). Another reproducible feature of our refined CSD procedure is the rarity of wounding: per experiment there were 1-2 surface wounds (skin abrasions) in 2-3 mice only, and deep bite wounds (skin penetration) did not occur at all, so that the majority of mice were wound free (see also (Azzinnari et al., 2014)).

3.2. Experiment A: A validated mouse model for social stress-induced hyperactive fear behaviour

The aims of this experiment were to investigate whether a model of social stress-induced excessive fear conditioning could be established and, if so, whether this effect could be reversed by the antidepressant-anxiolytic escitalopram as a demonstration of the predictive validity of the model. In addition, a first analysis of CSD effects on the kynurenine pathway was conducted.

3.2.1. CSD increased fear behaviour and escitalopram reversed this effect

Two days prior to CSD/CON, mice underwent an activity test (without CS or US) to determine baseline freezing levels. Percent time freezing was consistently low (mean = 8.0 %, range = 1-20 %; Fig. S1A); mice were allocated to CSD and CON groups by counterbalancing on this measure. CSD/CON was conducted on days 1-15, and within CSD and CON groups mice were then allocated to Drug groups and received either ESC or VEH on

days 15-18. At day 16 mice were given a second activity test (Fig. S1A): there was no Stress effect on % time freezing ($p=0.85$); there was a main effect of Drug ($F_{1,48}=5.71$, $p<0.03$) with ESC mice displaying lower freezing than VEH mice. Reduced freezing by ESC mice co-occurred with higher locomotor activity during non-freezing periods (data not shown), consistent with the reported effects of acute SSRI on mouse activity (Brocco et al., 2002). In CON but not in CSD mice, ESC also induced hyper-locomotion on days 17 and 18 (data not shown). At day 17, fear conditioning was conducted: For % time freezing in ITIs, there were main effects of ITI block ($F_{2,96}=11.82$, $p<0.0005$, Fig. S1A), Stress ($F_{1,48}=15.68$, $p<0.0005$, Fig. 2A) and Drug ($F_{1,48}=9.42$, $p<0.004$, Fig. 2A), respectively indicating that mice acquired increased ITI freezing as conditioning progressed, CSD mice displayed higher ITI freezing than CON mice, and ESC mice displayed lower ITI freezing than VEH mice. For % time freezing during CS, there were main effects of CS block ($F_{2,96}=14.41$, $p<0.0005$, Fig. S1B) and Stress ($F_{1,48}=19.32$, $p<0.0005$, Fig. 2B): mice acquired increased CS freezing as conditioning progressed, and CSD mice displayed higher CS freezing than CON mice. At day 18, fear expression, dependent on consolidation and recall of CS-US conditioning, was measured: For % time freezing in ITIs, there were main effects of ITI block ($F_{2,96}=5.37$, $p<0.006$, Fig. S1C), Stress ($F_{1,48}=4.30$, $p<0.05$, Fig. 2C) and Drug ($F_{1,48}=7.73$, $p<0.008$, Fig. 2C): mice expressed lower ITI freezing as the test progressed, CSD mice displayed higher ITI freezing than CON mice, and ESC mice displayed lower ITI freezing than VEH mice. For % time freezing during CS, there were main effects of Stress ($F_{1,48}=9.28$, $p<0.004$, Fig. 2D) and Drug ($F_{1,48}=4.77$, $p<0.05$, Fig. 2D): CSD mice displayed higher CS freezing than CON mice, and ESC mice displayed lower CS freezing than VEH mice.

Therefore, CSD did not affect freezing in a neutral arena but led to excessive learning and memory of a CS and context associated with a painful US. Daily escitalopram did not

affect fear learning but reduced fear memory, such that expression by CSD-ESC mice was similar to that of CON-VEH mice. Accordingly, this experiment demonstrates that CSD leads to a hyperactive fear system; that the excessive fear memory is attenuated by an antidepressant-anxiolytic drug provides evidence for predictive validity of this model.

(FIGURE 2 ABOUT HERE)

3.2.2. Evidence that CSD activated pro-inflammatory cytokines and kynurenine pathway

At day 20, a sample of the above CON-VEH and CSD-VEH mice was studied in terms of spleen weight, blood and brain levels of cytokines, kynurenines and monoamines. This *ex vivo* analysis was conducted to investigate whether previously reported effects of CSD in terms of increased plasma cytokine levels and splenomegaly (Azzinnari et al., 2014) could be substantiated and, in particular, as a first assessment of CSD effects on the KYN pathway.

CSD mice had larger spleens than CON mice in terms of absolute weight (CON 75.6±9.4 mg, CSD 93.1±16.8 mg, $t_{(14)}$=-2.58, p<0.03) and spleen weight/body weight (mg/g, CON 2.5±0.3, CSD 3.0±0.4, $t_{(14)}$=-2.88, p<0.02). Relative to CON mice, CSD mice had higher plasma levels of TNF-α (Fig. 3A) and IFN-γ (Fig. 3B). For each cytokine one CSD mouse had a high outlier value which was excluded from the *t*-test: TNF-α $t_{(12)}$=-2.33, p<0.05; IFN-γ $t_{(12)}$=-2.83, p<0.02 (with inclusion of all data in the (non-parametric) Mann-Whitney *U* test: TNF-α $U_{(8,7)}$=9.5, p<0.04; IFN-γ $U_{(8,7)}$=6, p<0.01). In ventral hippocampus (vHIPP) there was no CSD effect on TNF-α levels (CON: 0.63±0.19 pg/mg, CSD 0.64±0.18 pg/mg, p=0.68). In dorsal striatum (DS), all IFN-γ levels were below the assay LLOQ.

With regard to plasma kynurenines, relative to CON mice, CSD mice had higher KYN ($t_{(13)}$=3.17, p<0.01; Fig. 3C) and KYNA ($t_{(13)}$= 4.66, p<0.0005; Table 1), a borderline non-significant increase in 3-HK ($t_{(13)}$=1.99, p=0.07; Fig. 3D), similar levels in terms of QUIN

(p=0.40; Table 1) and TRP (p=0.91; Table 1), and lower levels of 5-HT ($t_{(12)}$=3.14, p<0.01; Table 1). In amygdala, CSD mice had higher tissue levels of KYN ($t_{(13)}$=4.34, p<0.0008; Fig. 3E) and 3-HK ($t_{(13)}$=6.60, p<0.0005; Fig. 3F). There was no CSD effect on amygdala TRP, 5-HT or DA levels (Table 1). The other brain regions analysed also demonstrated robust CSD-induced activation of the KYN pathway (Table 1): KYN was higher in CSD than CON mice in vHIPP, medial prefrontal cortex (mPFC) and dorsal raphe nucleus (DRN); 3-HK was higher in CSD than CON mice in vHIPP and DRN. As in amygdala, CSD was largely without effect on TRP and 5-HT levels in these brain regions. In vHIPP specifically, DA was higher in CSD than CON mice.

Therefore, this experiment replicates previous findings of CSD-induced splenomegaly and increased plasma TNF-α, and also demonstrates that CSD increases plasma IFN-γ and is without effect on brain levels of TNF-α and IFN-γ. Furthermore, it provides first evidence that CSD increases kynurenine pathway activity in terms of KYN and KYNA in plasma, and KYN in each of and 3-HK in most of the brain regions investigated. These mice were also exposed to aversive CS-US stress, which could confound effects of CSD per se. The effects of CSD on the kynurenine pathway without any potential confound were studied in Expt B.

(FIGURE 3 ABOUT HERE)

(TABLE 1 ABOUT HERE)

3.3. Experiment B: CSD increased kynurenines and IDO1 inhibitor reversed this effect

LC-MS/MS was used to investigate CSD effects on the KYN pathway in blood and brain, and whether any CSD-induced increases could be reversed by acute treatment with IDO1 inhibitor (IDOInh). CSD and CON mice were administered IDOInh or VEH on day 16, and blood and brain samples were collected 2 h later (Fig. 1).

Total plasma IDOInh level was similar in CON and CSD mice (Table 2) and also similar to that in the plasma exposure study (Fig. 4A); estimated unbound plasma level of IDOInh

was 1400±500 nM and therefore 3-4x IC_{50} for IDO1. For plasma KYN (Fig. 4B) there was a Stress x Drug interaction ($F_{1,27}$=8.12, p<0.009): CSD-VEH mice had higher KYN than each of the other groups, and CON-IDOInh and CSD-IDOInh mice had lower KYN than CON-VEH mice. For plasma 3-HK (Fig. 4C) there was also a Stress X Drug interaction ($F_{1,27}$=4.93, p<0.04): CSD-VEH mice had higher 3-HK than CSD-IDOInh mice. For plasma QUIN (Table 2) there was a main effect of Stress ($F_{1,27}$=5.87, p<0.03): CSD mice actually had lower levels than CON mice. For plasma KYNA (Table 2) there were no significant effects (p≥0.08;); therefore, in contrast to Expt A, KYNA was not increased in CSD-VEH versus CON-VEH mice. For plasma TRP (Table 2) there was a Stress X Drug interaction ($F_{1,27}$=7.26, p<0.02): CON-IDOInh mice had higher TRP than each of the other groups. There were no effects on 5-HT (Table 2).

In amygdala tissue samples, for KYN (Fig. 4D) there was a Stress X Drug interaction ($F_{1,27}$=5.95, p<0.03): CSD-VEH mice had higher KYN than each of the other groups, which had similar levels. For 3-HK (Fig. 4E), the level was below LLOQ in most CON and CSD-IDOInh mice whilst it was quantifiable in all CSD-VEH samples; the low LC-MS/MS sensitivity for 3-HK in this experiment precluded statistical analysis (cf. Expt A, Fig. 3F). Data for TRP, 5-HT and DA are given in Table 2. For TRP there was a main effect of Drug ($F_{1,27}$=6.37, p<0.02) and a borderline non-significant interaction (p=0.08): TRP was higher in CON-IDOInh mice than in each of the other groups. There were no effects for 5-HT (p≥0.19). For DA there was a main effect of Drug ($F_{1,27}$=8.38, p<0.009), with higher DA levels in mice that received IDOInh.

In vHIPP tissue samples, total IDOInh level in CON and CSD mice was 3-4 µmol/kg (Table 2) and therefore estimated unbound tissue levels were about 0.2x IC_{50} for IDO1. For KYN (Fig. 4F) there was a Stress X Drug interaction ($F_{1,27}$=4.48, p<0.04): CSD-VEH mice had higher KYN than each of the other three groups, which had similar levels. For 3-HK (Fig. 4G) there was a Stress X Drug interaction ($F_{1,27}$=4.212; p=0.05): CSD-VEH mice had higher 3-HK

than each of the other three groups, and CON-IDOInh mice had lower 3-HK than CON-VEH mice. For TRP (Table 2) there was a main effect of Drug ($F_{1,27}$=4.32, p<0.05), primarily due to higher levels in CON-IDOInh mice. For 5-HT and DA (Table 2) there were no significant effects.

Therefore, at day 16, KYN and 3-HK levels in blood, amygdala and vHIPP were higher in CSD than CON mice. These CSD effects were reversed by acute p.o. IDOInh. Whilst TRP levels in plasma, amygdala and vHIPP were similar in CSD and CON mice, IDOInh increased plasma and brain levels of TRP in CON mice specifically, and the increase in plasma TRP co-occurred with decreased plasma KYN in these CON mice. Amygdala and vHIPP 5-HT were similar in CSD-VEH and CON-VEH mice and unaffected by IDOInh. Amygdala and vHIPP DA were similar in CSD-VEH and CON-VEH mice, whilst IDOInh increased DA in amygdala.

(FIGURE 4 ABOUT HERE)

(TABLE 2 ABOUT HERE)

3.4. Experiment C: CSD without effect on Ido1/IDO1 expression and increased Tdo2 expression

3.4.1. Gene expression in splenocytes, ileum and liver

Using qPCR, effects of CSD on the expression of the genes encoding enzymes that catabolize TRP-KYN conversion were measured in splenic mononuclear cells (SMCs), ileum and liver (Table 3). For qPCR assays, final cDNA concentration was 2 ng/µl or, given the low expression of *Ido1* mRNA in liver, 5 ng/µl. Splenic MC expression of *Ido1* was not affected by CSD (p=0.82), while *Ido2* and *Tdo2* mRNAs were undetectable. In ileum, there was no effect of CSD on *Ido1* (p=0.87) or *Tdo2* (p=0.29) expression, while *Ido2* was barely detectable. CSD did

not affect *Ido1* (p=0.23) or *Ido2* (p=0.07) expression in liver; however, CSD mice had higher liver *Tdo2* mRNA expression than CON mice ($t_{(18)}$= -5.40, p<0.001).

(TABLE 3 ABOUT HERE)

3.4.2. IDO1 protein expression in ileum and liver

For ileum, a protein band with a size of ~45 kDa, corresponding to IDO1, was selectively detected (Fig. 5); as for mRNA, there was no effect of CSD on IOD1 protein expression (p=0.33). In liver, IDO1 protein expression was not detectable (Fig. 5), in line with previous findings (Dai and Zhu, 2010).

(FIGURE 5 ABOUT HERE)

3.5. Experiment D: CSD increased fear behaviour and IDO1 inhibitor reversed this effect

Using the CSD-hyperactive fear model established in Expt A, the aim of this experiment was to investigate whether the increased fear in CSD mice could be reversed by daily IDOInh administration, as was demonstrated for escitalopram.

In the activity test at two days prior to CSD/CON, % time freezing was consistently low (mean=8.3 %, range=2-24 %, Fig. S2A); mice were allocated to CSD and CON by counterbalancing on this measure. CSD/CON was conducted on days 1-15, and CSD and CON mice received either IDOInh or VEH on days 15-18. At day 16 mice were given a second activity test (Fig. S2A): there were no effects of Stress or Drug on % time freezing (p≥0.12) (also, there were no IDOInh effects on locomotor activity cf. escitalopram effect in Expt A). At day 17, fear conditioning was conducted: For % time freezing in ITIs, there were main effects of ITI block ($F_{2,104}$=27.68, p<0.0005, Fig. S2A) and Stress ($F_{1,52}$=12.34, p<0.001, Fig. 6A): mice acquired higher ITI freezing as conditioning progressed, and CSD mice acquired higher ITI freezing than CON mice. For % time freezing during CS, there was a Stress X CS block interaction ($F_{2,104}$=4.48, p<0.02, Fig. S2B) as well as main effects of CS block

($F_{2,104}$=13.67, p<0.0005) and Stress ($F_{1,52}$=9.32, p<0.004, Fig. 6B): CSD mice acquired progressively higher freezing than CON mice as conditioning progressed. At day 18, fear expression was tested: For % time freezing in ITIs, there was a Stress X Drug interaction ($F_{1,52}$=7.83, p<0.007, Fig. 6C): % time freezing was higher in CSD-VEH mice than CSD-IDOInh and CON-VEH mice, with the latter two groups having similar freezing levels. There was a non-significant trend to higher freezing in CON-IDOInh relative to CON-VEH mice (p=0.07). For % time freezing during CS, there was a Stress X Drug interaction ($F_{1,52}$=7.16, p<0.01, Fig. 6D): % time freezing was higher in CSD-VEH mice than CSD-IDOInh mice and CON-VEH mice, with the latter two groups exhibiting similar levels. There was a non-significant trend to higher freezing in CON-IDOInh relative to CON-VEH mice (p=0.09).

Therefore, the CSD induction of excessive fear learning and memory observed in Expt A was replicated. Furthermore, IDOInh exerted a similar effect to that obtained with escitalopram: there was no effect on the excessive CS-US fear learning in CSD mice, but IDOInh did reduce fear memory such that fear expression by CSD-IDOInh mice was similar to that of CON-VEH mice. In contrast, in CON mice there was a tendency for IDOInh to increase fear learning and memory.

(FIGURE 6 ABOUT HERE)

4. Discussion

4.1. A valid mouse model for psychosocial stress-induced hyperactive fear

In various psychiatric disorders, psychosocial stress is a major aetiological factor and excessive fear is a major psychopathology (Cuthbert and Insel, 2013; Disner et al., 2011; Kendler et al., 2003; Kessler, 1997). Mouse CSD resulted in excessive fear learning and memory of an aversive CS-US association, indicating that psychosocial stress induced

generalized hyper-reactivity to aversion. Pavlovian fear learning-memory is an important translational readout for several reasons, including detailed knowledge of the neurobiology of adaptive fear learning-memory in healthy humans (Fullana et al., 2015) and mice (Duvarci and Pare, 2014; LeDoux, 2000; Wolff et al., 2014), and evidence for increased fear conditioning in MDD (Nissen et al., 2010) and GAD (Greenberg et al., 2013; Lissek et al., 2015). Repeated escitalopram reversed the increased fear expression, specifically, in CSD mice, indicating that the model has predictive validity. Indeed, in healthy humans and MDD patients, acute and repeated escitalopram reduced amygdala reactivity to fearful facial stimuli (Godlewska et al., 2012; Harmer, 2013). Whilst it was not the aim of the current study to investigate SSRI effects on kynurenines, it is noteworthy that the SSRI sertraline did not affect blood kynurenines in MDD patients (Zhu et al., 2013) and there was no effect of citalopram on CSF kynurenines in rat (Toghi et al., 1995).

4.2. Psychosocial stress-induced activation of the kynurenine pathway

In mice, social aggression activates immune-inflammation, including splenomegaly and increased blood pro-inflammatory cytokines, blood monocytes and brain microglia (Azzinnari et al., 2014; Hodes et al., 2014; Savignac et al., 2011; Wohleb et al., 2011; Wohleb et al., 2013). The CSD refinements of teeth-trimming of aggressor mice and limiting attacks to 1 min/day prevent bite wounding ((Azzinnari et al., 2014), present study) and thereby increase confidence that it is psychosocial stress *per se* that activates immune-inflammation. Relative to CON mice, CSD mice demonstrated: in blood plasma, higher KYN (Expt A, B), 3-HK (Expt B, not A) and KYNA (Expt A, not B), no change in TRP or QUIN (Expt A, B), and lower 5-HT (Expt A, not B); in brain tissue, higher KYN and 3-HK in amygdala (Expt A, B), vHIPP (Expt A, B), mPFC (Expt A) and DRN (Expt A), higher DA in vHIPP (Expt A), with TRP and 5-HT largely

unaffected (Expt A, B). That a small number of CSD effects were specific to one experiment only could be attributable to fear conditioning (Expt A only) or the time interval to sample collection (day 20 in Expt A, day 16 in Expt B). This evidence for social stress activation of the KYN pathway adds significantly to previous studies: Acute exposure of mice to rat increased brain KYN at 1 and 4 weeks post-stress (Miura et al., 2011); mouse chronic unpredictable mild stress resulted in higher plasma KYN and brain 3-HK (Agudelo et al., 2014), or higher 3-HK in amygdala and striatum and lower 3-HK in cingulate cortex (Laugeray et al., 2010). Furthermore, the mouse data complement the evidence for activation of the KYN pathway in human stress-related psychiatric disorders, most notably MDD to date. For example, relative to healthy controls, MDD patients have increased blood KYN (Bay-Richter et al., 2015; Kim et al., 2012; Sublette et al., 2011), and the density of QUIN-positive microglia was increased in anterior cingulate cortex in MDD patients (Steiner et al., 2011) (for review: (Reus et al., 2015)).

Plasma TNF-α and IFN-γ were moderately elevated in CSD mice, and synergistic effects of these two cytokines are essential for inflammation-induced increased *Ido1* expression in mouse (O'Connor et al., 2009a). In the present study however, CSD did not affect *Ido1*, or *Ido2*, mRNA expression or IDO1 protein expression, in spleen, ileum or liver. This was despite spleen and ileum being two of the major *Ido1*-expressing tissues (Dai and Zhu, 2010), and despite splenomegaly. *Tdo2* expression in liver, the major *Tdo2*-expressing tissue, was increased in CSD mice. Rats exposed to repeated physical restraint exhibit increased liver TDO, brain cortex *Tdo2* mRNA expression, and plasma KYN (Gibney et al., 2014). Mice exposed to acute acoustic + restraint stress exhibited increased *Ido1* mRNA expression in spleen, ileum and brain, dependent on increased plasma TNF-α and IFN-γ, that was apparent at 12 h after stress termination and returned to basal expression after 24 h

(Kiank et al., 2010). Whether CSD leads to transient increases in *Ido1* expression following the daily attack sessions remains to be investigated.

4.3. IDO1 inhibition blocks kynurenine pathway activation and excessive fear expression

Despite the absence of evidence for CSD induction of IDO1 expression, a causal pathway linking CSD, IDO1-dependent activation of the KYN pathway and increased fear behaviour was investigated. Firstly, acute IDOInh did indeed reverse the CSD-induced increase in KYN and 3-HK in blood and brain (amygdala, vHIPP). It was without effect on brain 5-HT and led to increased DA in the amygdala of CSD and CON mice. Second, repeated IDOInh administration was without effect on the excessive fear-freezing by CSD mice at CS-US conditioning, but on the following day it reduced the fear-freezing elicited by the CS to CON levels. These findings suggest that stress-induced IDO1-KYN pathway hyperactivity does not contribute to aversive learning but that it does stimulate the subsequent short- and long-term memory processes - consolidation and/or recall - that determine fear expression to the aversive CS, in mice that are hyper-reactive to aversive stimuli. IDOInh did not reduce the adaptive fear expression of CON mice, perhaps because KYN-pathway activity in non-stressed mice is below a threshold necessary to contribute to fear-memory formation. Indeed, IDOInh actually caused borderline increases in fear learning-memory in CON mice. Plasma KYN was sub-normal and plasma and brain TRP were supra-normal in CON-IDOInh mice, suggesting that basal TRP-KYN pathway activity is necessary for adaptive functioning (Schwarcz et al., 2012). A basal KYN-pathway function that might have been deficient in CON-IDOInh mice is inhibition of T-cell proliferation/autoimmunity (Julliard et al., 2014; Munn et al., 2002); also, their high TRP levels might have led to oxidative stress (Feska et al., 2006).

Therefore, the findings indicate that pharmacological normalization of stress-induced KYN pathway hyperactivity by IDO1 inhibition normalizes memory processing of aversive stimuli, and to a similar extent but via a different mechanism to that of escitalopram. With regard to mechanism-of-action on memory processes, both 3-HK and QUIN can induce oxidative stress and excitotoxicity in neurons, particularly when elevated chronically (Chiarugi et al., 2001; Haroon et al., 2012; Schwarcz et al., 2012). A limitation of the current study, given their important effects at several neurotransmitter-receptor types, was that QUIN and KYNA could not be measured in small brain-tissue samples. For example, QUIN and KYNA are agonistic and antagonistic, respectively, at the glutamate NMDA receptors which are major determinants of the synaptic plasticity processes underlying the different stages of learning and memory, including in amygdala (for review: (Maren and Quirk, 2004)).

Taken together, the current findings of CSD-induced TRP-KYN pathway hyperactivity, lack of CSD effect on *Ido1*/IDO1 expression, and complete normalization of CSD-induced TRP-KYN pathway hyperactivity by a specific IDO1 inhibitor, are consistent with CSD impacting on factors that regulate the post-translational activity of IDO1. These include pro-inflammatory cytokines, and factors governing the synthesis of the haem prosthetic group that is essential for the formation of the active IDO1 holoenzyme (Yeung et al., 2015). These post-translational factors constitute novel targets for regulating stress-induced increased IDO1-KYN pathway activity. The IDO1 enzyme catalyzes TRP-KYN pathway activity in periphery and brain, and TRP, KYN and 3-HK are also transported actively across the blood-brain-barrier via a large neutral amino acid transporter (Fukui et al., 1991). Regarding the site-of-action of IDOInh on the KYN pathway, the *in vitro* and *in vivo* data indicate that its main effect is in the periphery: the estimated protein-unbound IDOInh exposure in blood was 1.8x and in CSF only 0.2x its IC_{50} for IDO1. Given that 60 % of brain KYN is derived from

blood (Gal and Sherman, 1978; Schwarcz et al., 2012), reduced TRP-KYN-3HK conversion in the periphery of CSD-IDOInh mice would reduce KYN and 3-HK availability for brain transport, and also normalize their brain KYN and 3-HK, therefore. Whether low-level IDOInh activity in the brain microglia/infiltrating macrophages of CSD mice also occurs will require future study, as will the effects of compounds with high brain exposure. The interesting finding that CSD up-regulated liver *Tdo2* expression is unlikely to be relevant to the CSD-induced activation of the KYN pathway: the increase in KYN-pathway activity was reversed completely by IDOInh, and IDOInh activity against TDO2 is extremely low ((Liu et al., 2010), see section 2.4). However, future studies, for example with *Tdo2* knockout mice, will be needed to confirm this.

4.4. Integrating current findings with existing evidence for KYN-pathway effects on aversion processing

Studies to-date have focused on prophylactic effects of IDO1 inhibition. Pre-treatment of mice with the competitive IDO inhibitor 1-methyltryptophan (1-MT) decreased the effect-severity of repeated acoustic + restraint stress on physical condition and motor inactivity (Kiank et al., 2010). Pre-treatment with 1-MT prevented systemic LPS, which activates the IDO1-KYN pathway, from decreasing mouse activity in FST and TST (O'Connor et al., 2009b). Chronic co-treatment of rats with the TDO2 inhibitor allopurinol prevented restraint stress from decreasing FST activity (Gibney et al., 2014). Now the current study provides some of the first evidence for the reversal of behavioural effects of stress-induced IDO1-KYN pathway activation. Given the importance of amygdala and hippocampus in fear conditioning (Duvarci and Pare, 2014; LeDoux, 2000; Maren et al., 2013; Wolff et al., 2014) and stress-related psychiatric disorders (Harmer, 2013; Phelps and LeDoux, 2005; Price and Drevets, 2010;

Sheline et al., 2001), it is noteworthy that amygdala and vHIPP, together with other brain regions, exhibited increased levels of kynurenines. In healthy humans, LPS induced increased amygdala reactivity to fearful faces (Inagaki et al., 2012). Amygdala volume was reduced in MDD patients with reduced KYNA/QUIN plasma ratios (Savitz et al., 2015). Despite the rodent evidence for similar effects of psychosocial stress and acute immune-inflammatory challenge in some behavioural tests, it is important to note that in the case of Pavlovian fear conditioning, challenges such as LPS (Pugh et al., 1998; Thomson and Sutherland, 2005) and CD40 agonist antibody (Cathomas et al., 2015) actually decrease fear learning-memory whereas, as demonstrated here, psychosocial stress leads to increased fear learning-memory. Therefore, it will be essential to study and compare the immuno-neuro-behavioural effects of these two manipulations, and relate the findings to the aetio-pathophysiologies of negative valence psychopathologies in psychiatric and autoimmune disorders.

In summary, the present mouse study combines psychosocial stress, fear conditioning, biochemical analysis and an IDO1 inhibitor tool compound, to demonstrate that chronic social stress activation of the IDO1-KYN pathway in blood and brain contributes to fear memory formation. This model can be further applied to study stress effects on IDO1 activity, the neurobiological mechanism of KYN pathway-mediated consolidation of fear memory, and also whether other immune pathways contribute to the excessive fear learning exhibited by socially-stressed mice. As such, this study adds to the evidence that a causal pathway exists between psychosocial stress, immune-inflammation and the excessive reactivity to aversive stimuli that is a major domain in the stress-related neuropsychiatric disorders.

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Figure Captions

Figure 1. Experimental designs. Four inter-related experiments were conducted with mice exposed to chronic social defeat (CSD) or control handling (CON) on days 1-15. Mice were handled prior to experiments and body weights were measured daily. Each experiment was conducted with a different cohort of mice. Experiment A: Mice were given a test of baseline freezing in a neutral arena (BNA) and allocated to CSD and CON to counterbalance on this measure. A 3-day test paradigm of freezing in a neutral arena (NA) and during tone-electroshock (CS-US) fear conditioning (FC) and at fear expression (FE), was conducted on days 16, 17 and 18, respectively. Escitalopram (ESC) or vehicle (VEH) was administered by oral gavage (p.o.) after CSD on day 15 and at 1 hour pre-test on days 16-18. On day 20, mice were sacrificed (S) and physiological samples were collected from CON-VEH and CSD-VEH mice for determination of cytokines, tryptophan, kynurenines and monoamines. Experiment B: On day 16, IDO1 inhibitor (IDOInh) or VEH were administered p.o., followed 2 hours later by collection of physiological samples for determination of IDOInh, tryptophan, kynurenines and monoamines. Experiment C: On day 16, tissues were collected for determination of *Ido1*, *Ido2* and *Tdo2* mRNA expression and IDO1 protein expression. Experiment D: The 3-day freezing test paradigm was conducted on days 16-18, and IDOInh or VEH were administered p.o. after CSD on day 15 and at 2 hours pre-test on days 16-18.

Figure 2. Experiment A, effects of 15-day chronic social defeat versus control and repeated escitalopram (10 mg/kg p.o.) versus vehicle on fear conditioning on day 17 (A, B) and fear expression on day 18 (C, D). (A) Mean+SE % time freezing during inter-trial intervals (5x CS-US ITIs, 120 sec) in fear conditioning. (B) Mean+SE % time freezing to a tone stimulus (6x 20 sec CS + 2 sec x 0.15 mA electroshock US) in fear conditioning. (C) Mean+SE % time freezing

during ITIs (8x 90-sec) in fear expression. (D) Mean+SE % time freezing during CS (9x 30-sec tone) in fear expression. Figure S1 presents the data according to trial blocks (see Supplementary Material).

P values are for Stress X Drug X Trial block 3-way ANOVA: Stress main effect: * p<0.05, ** p<0.01, **** p<0.0005; Drug main effect: # p<0.05, ## p<0.01.

Figure 3. Experiment A, effects of 15-day chronic social defeat versus control on day-20 pro-inflammatory cytokines TNF-α and IFN-γ in plasma and kynurenine pathway metabolites in plasma and amygdala. Data are expressed as scatter plots and means for CON-VEH (N=8) and CSD-VEH (N=7) mice. (A) Plasma TNF-α, (B) Plasma IFN-γ, (C) Plasma kynurenine, (D) Plasma 3-OH-kynurenine, (E) Amygdala kynurenine, (F) Amygdala 3-OH-kynurenine.

P values are for Mann-Whitney *U* (A, B) or Student's *t*-test (C-F): *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 4. Experiment B, effects of 15-day chronic social defeat versus control and acute IDO inhibitor (200 mg/kg p.o.) versus vehicle on day-16 kynurenine and 3-OH-kynurenine in plasma and limbic brain regions. (A) Plasma PK profile of IDOInh after administration of 200 mg/kg p.o.; values are mean ± range (N=3). In (B)-(G) data are expressed as scatter plots and means for (B) Plasma kynurenine, (C) Plasma 3-OH-kynurenine, (D) Amygdala kynurenine, (E) Amygdala 3-OH-kynurenine, (F) ventral Hippocampal kynurenine, (G) ventral Hippocampal 3-OH-kynurenine . N=8 (CON-VEH, CON-IDOInh, CSD-IDOInh) or N=7 (CSD-VEH).

P values are for ANOVA and *post hoc* least significant difference test: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001

Figure 5. Experiment C, Western blot analysis of IDO protein in ileum and liver tissue. The panels show representative IDO bands for CON and CSD mice in ileum, whilst the protein was undetectable in liver. The histogram depicts relative IDO expression in ileum (mean+SE). *P* value is for Student's *t*-test.

Figure 6. Experiment D, effects of 15-day chronic social defeat versus control and repeated IDO inhibitor (200 mg/kg p.o.) versus vehicle on fear conditioning on day 17 (A, B) and fear expression on day 18 (C, D). (A) Mean+SE % time freezing during inter-trial intervals (5x CS-US ITI, 120 sec) in fear conditioning. (B) Mean+SE % time freezing to a tone stimulus (6x 20 sec CS + 2 sec x 0.15 mA electroshock US) in fear conditioning. (C) Mean+SE % time freezing during ITIs (8x 90-sec) in fear expression. (D) Mean+SE % time freezing during CS (9x 30-sec) in fear expression. Figure S2 presents the data according to trial blocks (see Supplementary Material).

P values are for Stress X Drug X Trial block 3-way ANOVA and *post hoc* least significant difference test: Stress main effect or *post hoc* effect following Stress X Drug interaction: * $p<0.05$, ** $p<0.01$, ***$p<0.001$, **** $p<0.0005$. Drug *post hoc* effect following Stress X Drug interaction: # $p<0.05$.

FIGURE 1

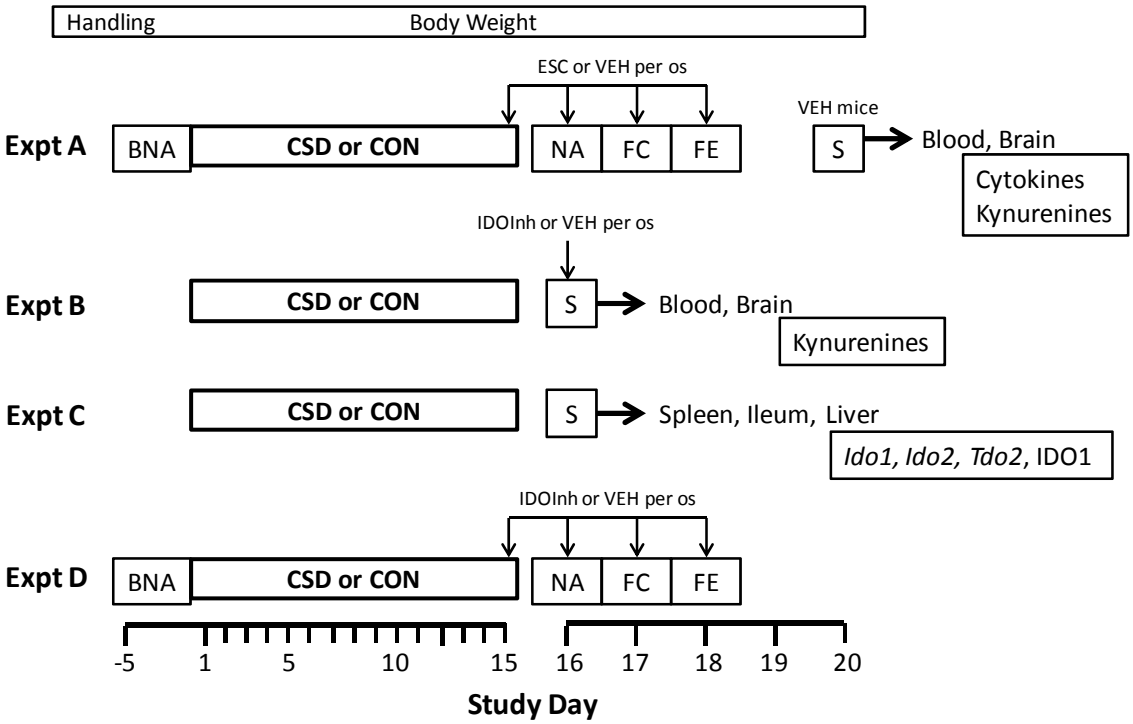
Handling
Body Weight
ESC or VEH per os
Expt A
BNA
CSD or CON
NA
FC
FE
VEH mice
S
Blood, Brain
Cytokines
Kynurenines
IDOInh or VEH per os
Expt B
CSD or CON
S
Blood, Brain
Kynurenines
Expt C
CSD or CON
S
Spleen, Ileum, Liver
Ido1, Ido2, Tdo2, IDO1
IDOInh or VEH per os
Expt D
BNA
CSD or CON
NA
FC
FE
-5
1
5
10
15
16
17
18
19
20
Study Day

FIGURE 2

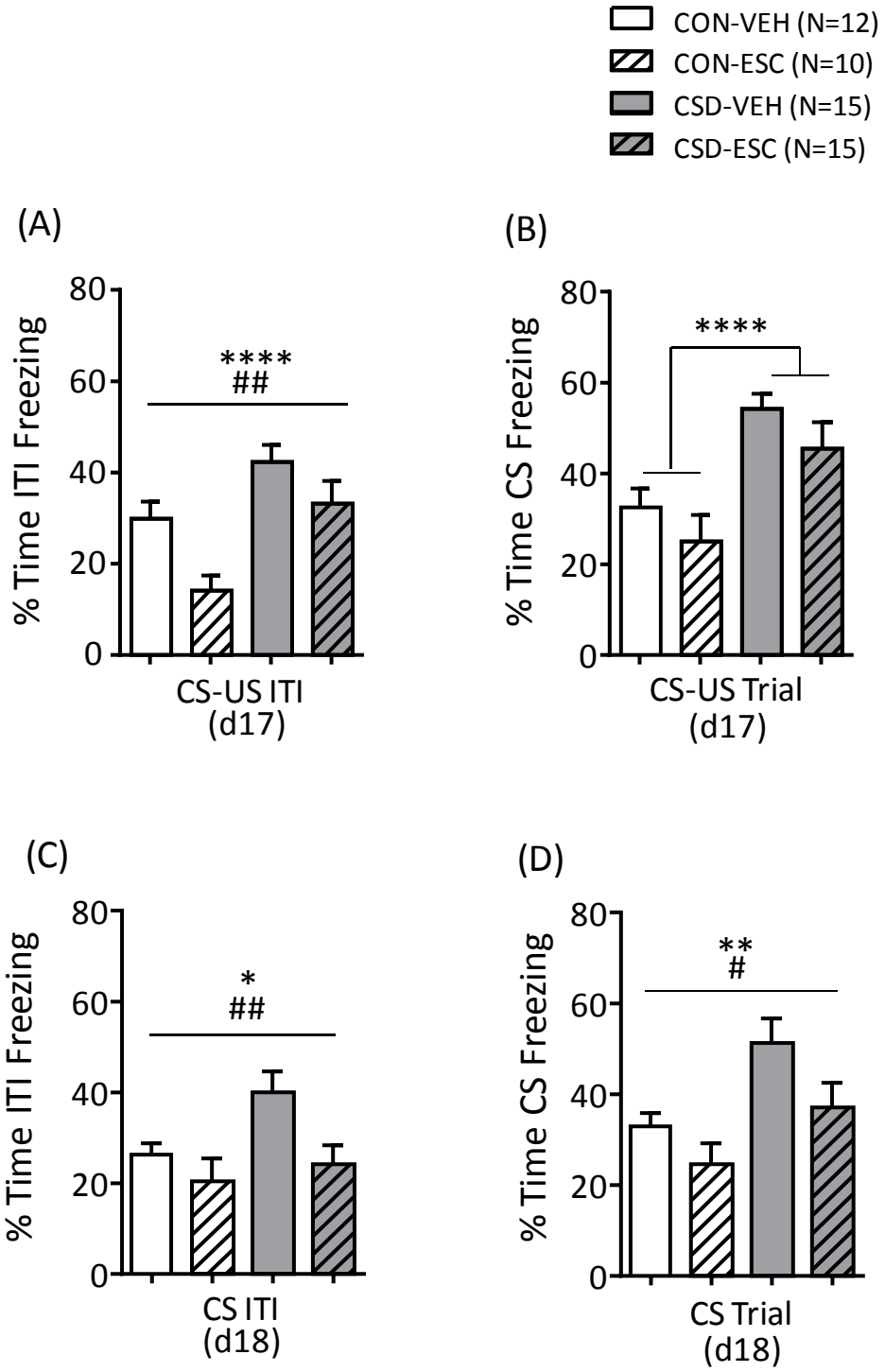
CON-VEH (N=12)
CON-ESC (N=10)
CSD-VEH (N=15)
CSD-ESC (N=15)
(A)
% Time ITI Freezing
80
60
40
20
0
****
##
CS-US ITI
(d17)
(B)
% Time CS Freezing
****
CS-US Trial
(d17)
(C)
% Time ITI Freezing
*
##
CS ITI
(d18)
(D)
% Time CS Freezing
**
#
CS Trial
(d18)

FIGURE 3

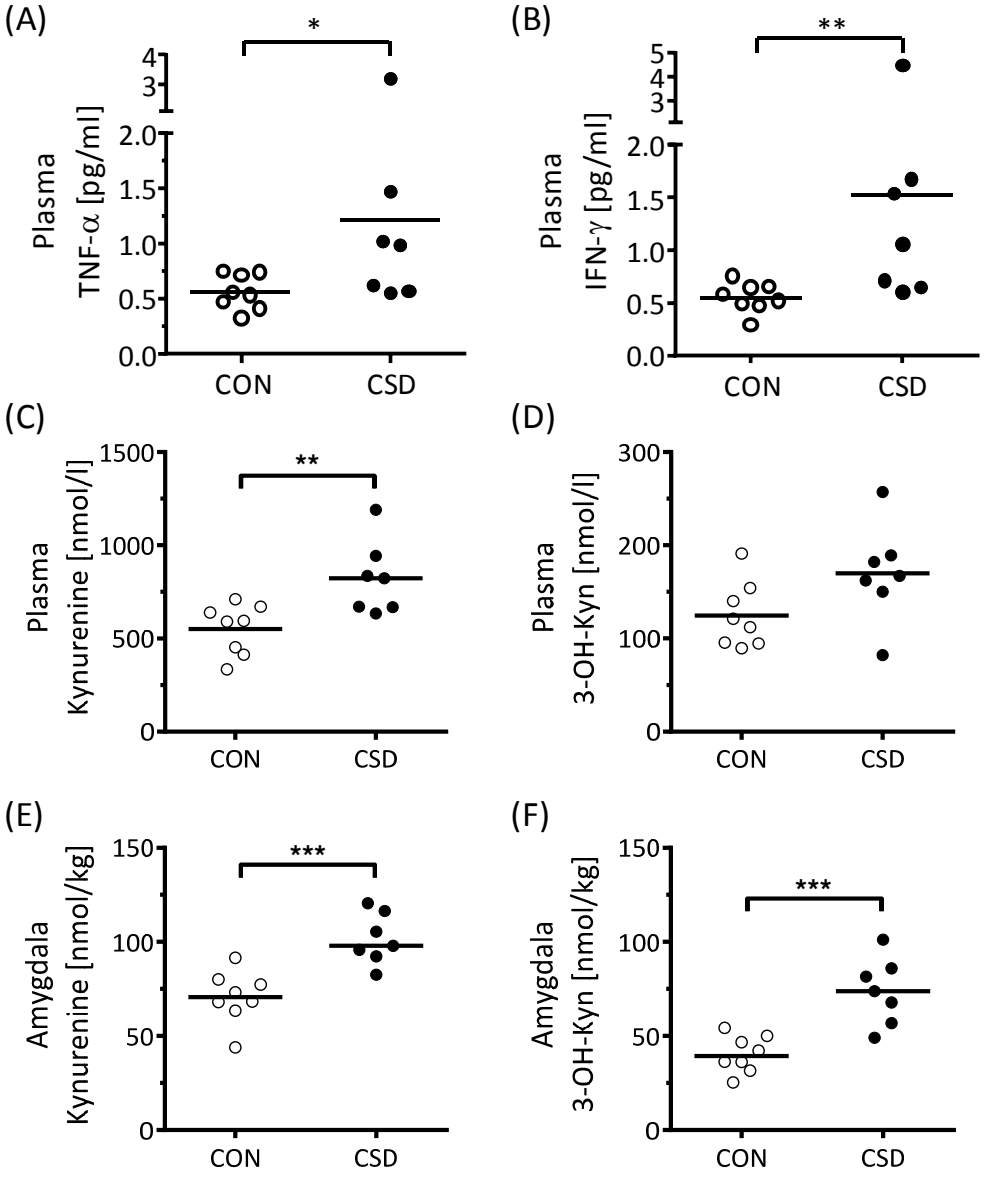
(A)
Plasma TNF-α [pg/ml]
*
CON
CSD
(B)
Plasma IFN-γ [pg/ml]
**
CON
CSD
(C)
Plasma Kynurenine [nmol/l]
**
CON
CSD
(D)
Plasma 3-OH-Kyn [nmol/l]
CON
CSD
(E)
Amygdala Kynurenine [nmol/kg]
***
CON
CSD
(F)
Amygdala 3-OH-Kyn [nmol/kg]
***
CON
CSD

FIGURE 4

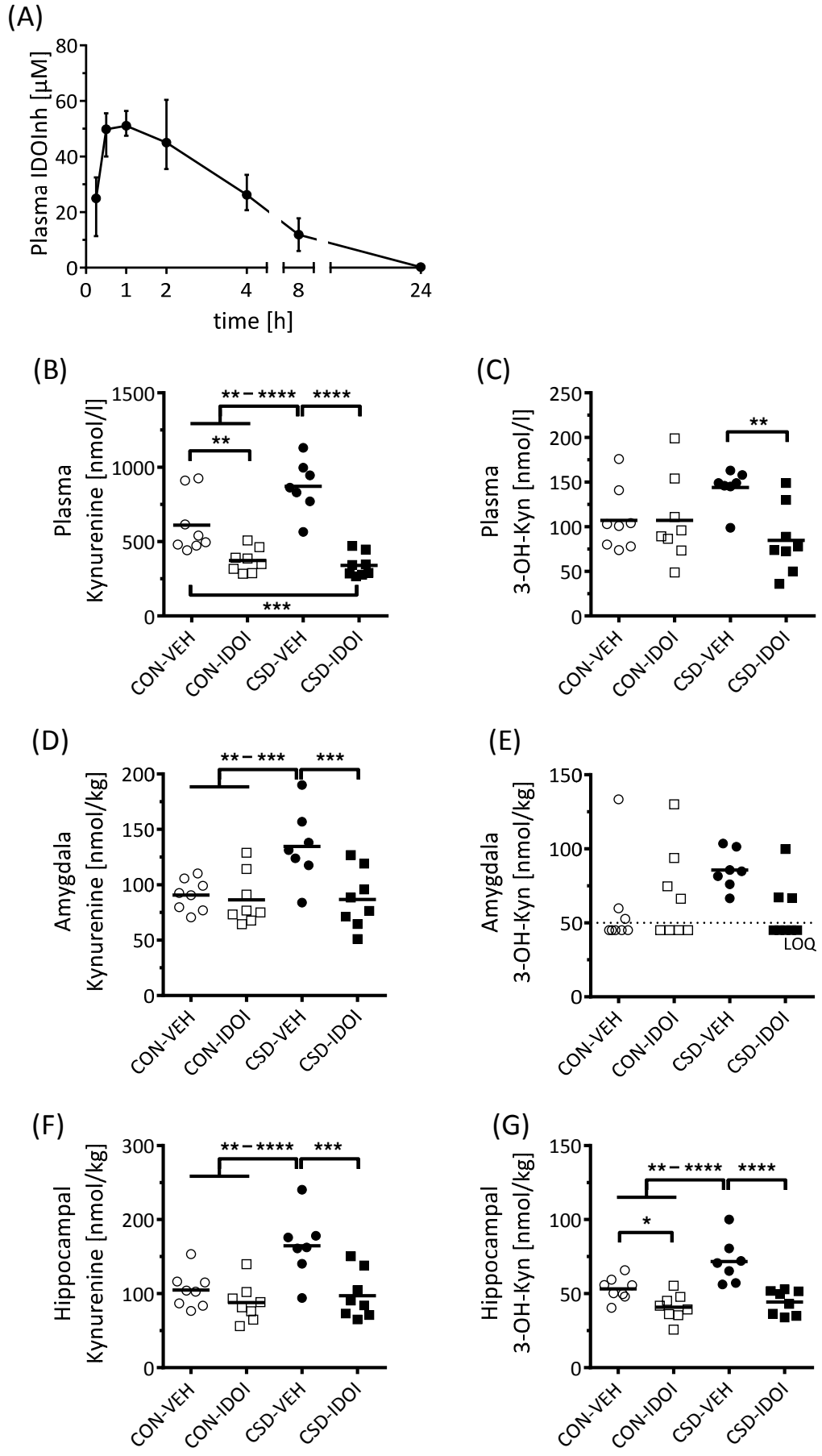
(A)
Plasma IDOInh [µM]
80
60
40
20
0
0 1 2 4 8 24
time [h]
(B)
Plasma
Kynurenine [nmol/l]
1500
1000
500
0
** **** ****
**
***
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI
(C)
Plasma
3-OH-Kyn [nmol/l]
250
200
150
100
50
0
**
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI
(D)
Amygdala
Kynurenine [nmol/kg]
200
150
100
50
0
** *** ***
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI
(E)
Amygdala
3-OH-Kyn [nmol/kg]
150
100
50
0
LOQ
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI
(F)
Hippocampal
Kynurenine [nmol/kg]
300
200
100
0
** **** ***
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI
(G)
Hippocampal
3-OH-Kyn [nmol/kg]
150
100
50
0
** **** ****
*
CON-VEH
CON-IDOI
CSD-VEH
CSD-IDOI

FIGURE 5

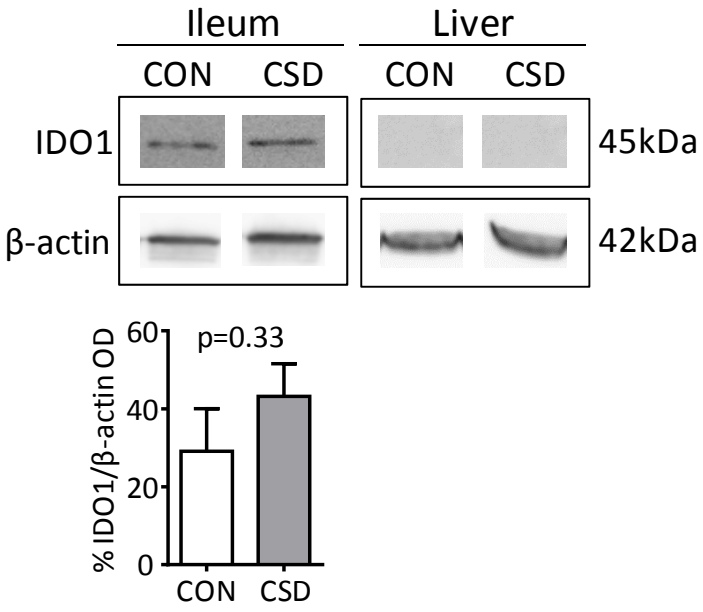
Ileum
Liver
CON
CSD
CON
CSD
IDO1
45kDa
β-actin
42kDa
% IDO1/β-actin OD
60
40
20
0
p=0.33
CON
CSD

FIGURE 6

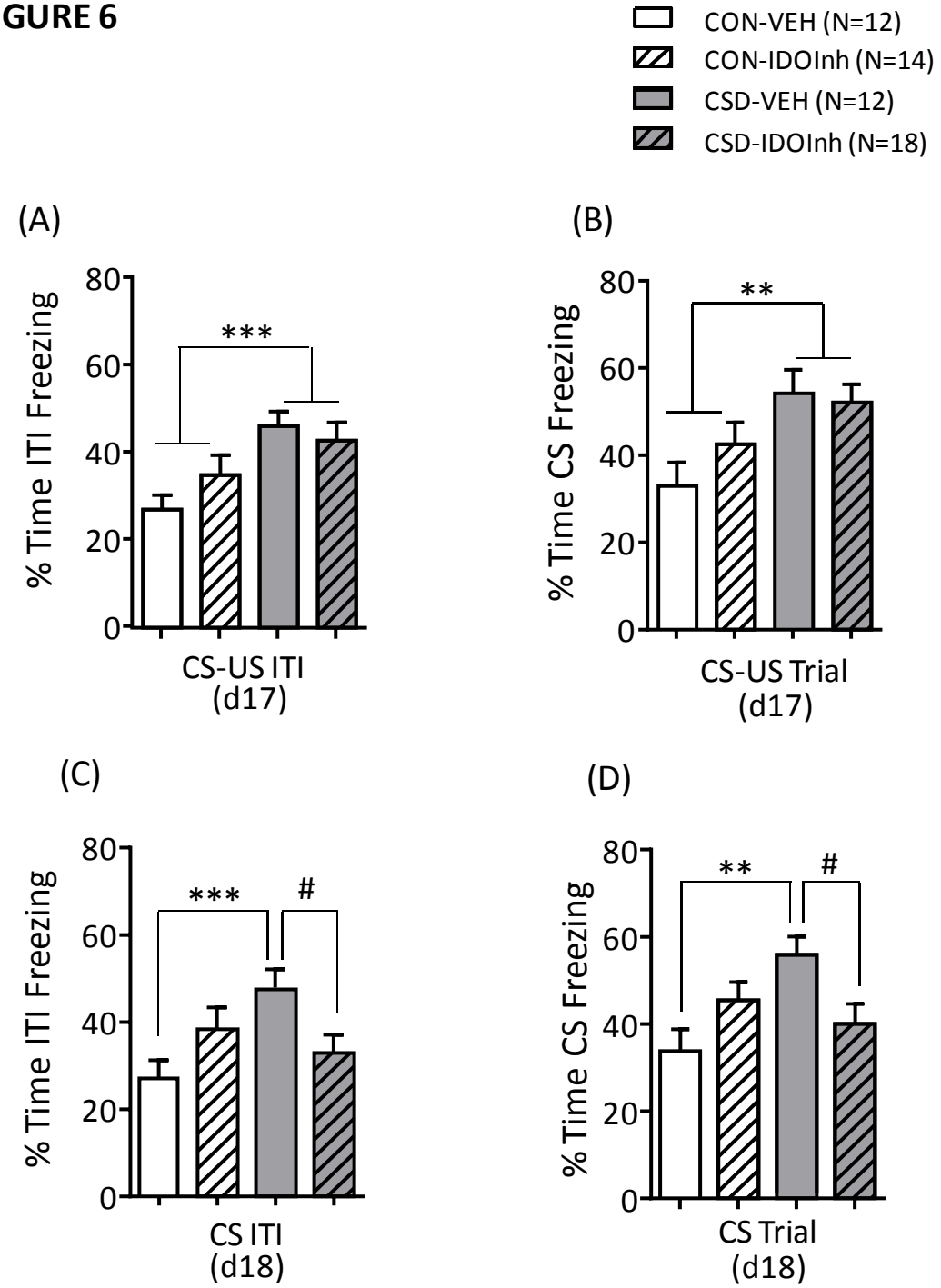
CON-VEH (N=12)
CON-IDOInh (N=14)
CSD-VEH (N=12)
CSD-IDOInh (N=18)
(A)
% Time ITI Freezing
80
60
40
20
0
***
CS-US ITI
(d17)
(B)
% Time CS Freezing
**
CS-US Trial
(d17)
(C)
% Time ITI Freezing
***
#
CS ITI
(d18)
(D)
% Time CS Freezing
**
#
CS Trial
(d18)

Table 1. Effects of chronic social defeat on tryptophan, kynurenines and monoamines in plasma and brain regions (Experiment A)

Metabolite	Group	Plasma [nmol/l]	Amygdala [nmol/kg]	v. Hippocampus [nmol/kg]	Dorsal raphe nucleus [nmol/kg]	Medial prefrontal cortex [nmol/kg]
Tryptophan	CON	89±8 µM	15.8±1.4 µM	16.0±1.4 µM	6.9±0.8 µM	14.3±1.1 µM
	CSD	88±6 µM	15.1±1.2 µM	15.1±1.5 µM	10.2±1.6 µM	14.3±1.2 µM
Kynurenine	CON	Figure 2		104±5	56±5	91±7
	CSD			143±9**	112±6****	132±9**
3-OH-kynurenine	CON	Figure 2		68±2	45±5	60±6
	CSD			96±8**	63±6*	81±8
Serotonin	CON	6.8±1.1 µM	3.6±0.2 µM	3.8±0.3 µM	4.6±0.3 µM	1.4±0.1 µM
	CSD	2.4±0.6 µM**	3.8±0.4 µM	4.0±0.3 µM	4.2±0.6 µM	1.4±0.2 µM
Dopamine	CON	n.d.	14±4 µM	0.08±0.01 µM	0.32±0.06 µM	3.0±1.0 µM
	CSD	n.d.	21±6 µM	0.13±0.06 µM*	0.29±0.08 µM	2.5±0.7 µM
Quinolinic acid	CON	318±50		below LLOQ in brain tissue samples		
	CSD	263±31				
Kynurenic acid	CON	43±5		below LLOQ in brain tissue samples		
	CSD	72±4***				

Values are mean±SE, CON=8 and CSD=7; * p<0.05, ** p <0.01, *** p<0.001, **** p<0.0001 in *t*-test. n.d. = not detectable

Table 2. Effects of chronic social defeat and IDO inhibitor on tryptophan, kynurenines and monoamines in plasma and brain regions (Experiment B)

Region	Metabolite	CON-VEH (N=8)	CON-IDOInh (N=8)	CSD-VEH (N=7)	CSD-IDOInh (N=8)
Plasma					
	IDO-Inhibitor [µmol/l]	n.d.	45±5	n.d.	41±7
	Tryptophan [µmol/l]	114±8	137±7*	108±6$^{\#}$	89±10*$^{;\#\#\#}$
	Serotonin [µmol/l]	4.9±1.0	7.5±2.0	4.6±1.3	4.3±1.1
	Quinolinic acid [nmol/l]	120±15	167±39	98±6	78±11$^{\#}$
	Kynurenic acid [nmol/l]	65±7	92±13	72±7	61±11
Amygdala					
	Tryptophan [µmol/kg]	16.3±0.5	25.3±3.8**	15.6±0.2$^{\#\#}$	17.9±1.9$^{\#}$
	Serotonin [µmol/kg]	5.1±0.3	5.5±0.2	5.7±0.1	5.5±0.3
	Dopamine [µmol/kg]	25.4±5.1	45.7±5.3**	33.3±4.5	41.3±4.2*
v. Hippocampus					
	IDO-Inhibitor [µmol/kg]	n.d.	4.4±0.7	n.d.	3.2±0.6
	Tryptophan [µmol/kg]	14.4±0.5	22.6±3.8*	14.0±0.6$^{\#}$	15.5±2.2$^{\#}$
	Serotonin [µmol/kg]	6.5±0.2	6.1±0.5	6.1±0.3	6.0±0.3
	Dopamine [µmol/mg]	0.06±0.01	0.07±0.01	0.09±0.01	0.06±0.01

Values are mean±SE.

In ANOVA followed by pair-wise comparisons: * $p<0.05$ versus CON-VEH, $^{\#}$ $p<0.05$ versus CON-IDOInh, $^{\#\#}$ $p<0.01$ versus CON-IDOInh, $^{\#\#\#}$ $p<0.001$ versus CON-IDOInh. n.d. = not detectable

Table 3. Effects of chronic social defeat on mRNA expression of kynurenine pathway enzymes

	Splenic MCs		Ileum intestine		Liver	
	CON	CSD	CON	CSD	CON	CSD
Ido1	0.34 ± 0.52	0.42 ± 0.88	0.11 ± 0.19	0.05 ± 0.72	-0.64 ± 0.61	-0.30 ± 0.60
Ido2	n.d.	n.d.	n.d./Ct>36	n.d./Ct>36	0.43 ± 0.41	0.94 ± 0.73
Tdo2	n.d.	n.d.	1.10 ± 0.96	0.41 ± 0.96	-0.09 ± 0.40	0.99 ± 0.49[a]

Splenic MCs = Splenic mononuclear cells; n.d. = not detectable indicating low expression.

Scores are $\log_2$-transformed normalized expression values (mean ± SD).

a p<0.001 in t-test, CSD vs CON.

Normalization factor for each tissue type and gene was the average of the Ct values of the reference genes, *Hprt1* and *Tbp*.

Supplementary Material

Table S1. List of primer sequences used in this study

Gene	Primer F (5'->3')	Primer R (5'->3')
Hprt1	CTTCCTCCTCAGACCGCTTT	TTTTCCAAATCCTCGGCATA
Tbp	CATCTCAGCAACCCACACAG	GGGGTCATAGGAGTCATTGG
Ido1	CAAAGCAATCCCCACTGTATCC	ACAAAGTCACGCATCCTCTTAAA
Ido2	CCTCATCCCTCCTTCCTTTC	GGAGCAATTGCCTGGTATGT
Tdo2	AGGAACATGCTCAAGGTGATAGC	CTGTAGACTCTGGAAGCCTGAT

Abbreviations: Hprt1, hypoxanthine phosphoribosyltransferase 1; Tbp, TATA box binding protein; Ido1, 2, indoleamine 2,3-dioxygenase 1,2; Tdo2, tryptophan 2,3-dioxygenase 2.

FIGURE S1

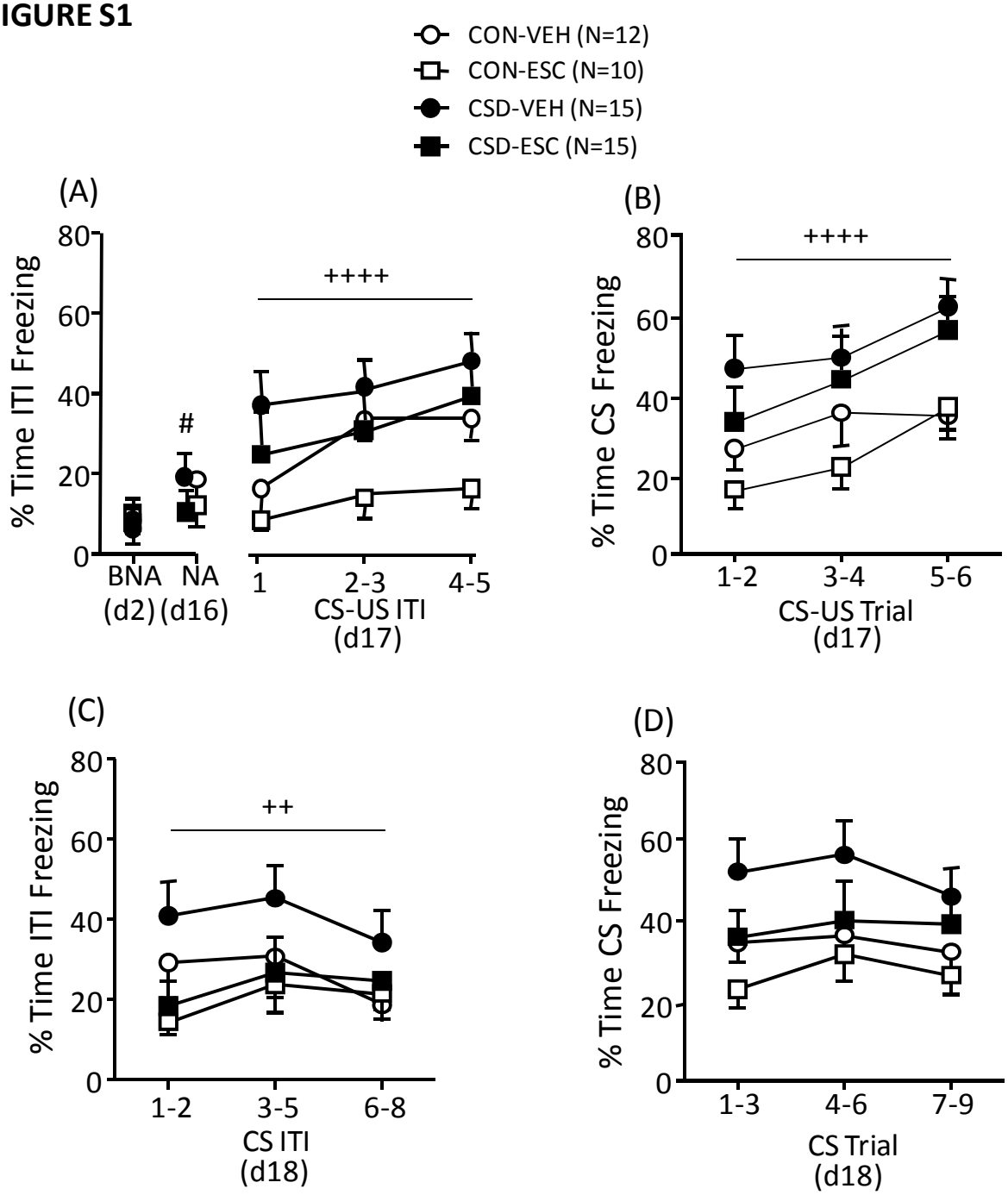

Figure S1. Experiment A, effects of 15-day chronic social defeat versus control and repeated Escitalopram (10 mg/kg p.o.) versus vehicle on freezing in a neutral arena (A), in fear conditioning (A, B) and in fear expression (C, D), on days 16, 17 and 18, respectively. (A) Mean+/-SE % time freezing in the 15-min baseline neutral arena test on day -2 (BNA), the 15-min neutral arena test (NA), and during blocks of inter-trial intervals (CS-US ITIs, 120 sec) in fear conditioning. Fear conditioning data are given in blocks of 1-2 ITI. (B) Mean+/-SE % time freezing to a tone stimulus (20 sec CS + 2 sec x 0.15 mA electroshock US) in fear conditioning; data are given in blocks of 2 CS. (C) Mean+/-SE % time freezing during 90-sec ITI in fear expression; data are given in blocks of 2-3 ITI. (D) Mean+/-SE % time freezing during 30-sec CS in fear expression; data are given in blocks of 3 CS. Figure 2 presents the data collapsed across trial blocks

P values are for Stress X Drug X Trial block 3-way ANOVA and *post hoc* least significant difference test: Trial block main effect: ++ $p<0.01$, ++++ $p<0.0005$.

FIGURE S2

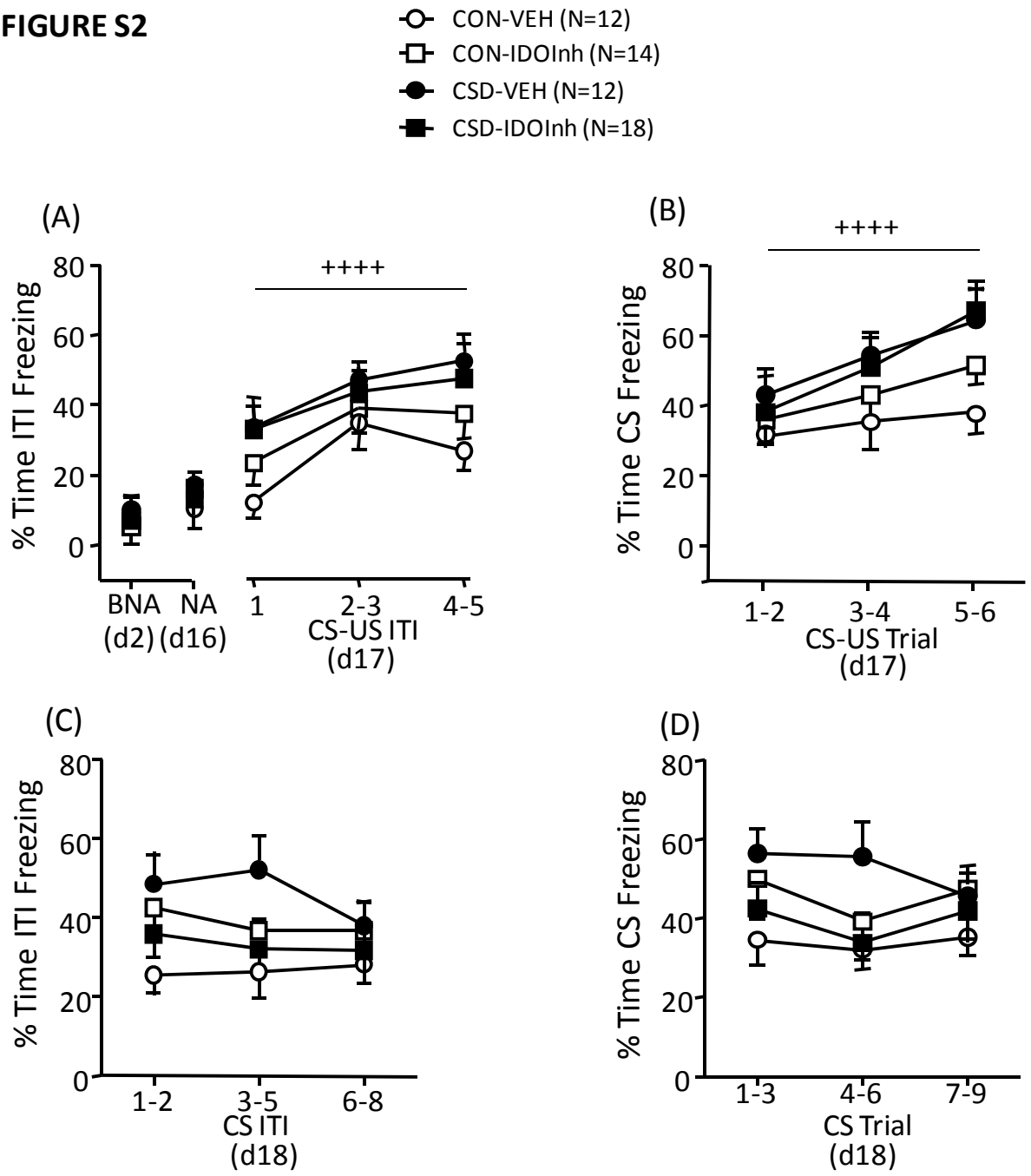

Figure S2. Experiment D, effects of 15-day chronic social defeat versus control and repeated IDO inhibitor (200 mg/kg p.o.) versus vehicle on freezing in a neutral arena (A), in fear conditioning (A, B) and in fear expression (C, D), on days 16, 17 and 18, respectively. (A) Mean+/-SE % time freezing in the 15-min baseline neutral arena test on day -2 (BNA), the 15-min neutral arena test (NA), and during blocks of inter-trial intervals (CS-US ITIs, 120 sec) in fear conditioning. Fear conditioning data are given in blocks of 1-2 ITI. (B) Mean+/-SE % time freezing to a tone stimulus (20 sec CS + 2 sec x 0.15 mA electroshock US) in fear conditioning; data are given in blocks of 2 CS. (C) Mean+/-SE % time freezing during 90-sec ITI in fear expression; data are given in blocks of 2-3 ITI. (D) Mean+/-SE % time freezing during 30-sec CS in fear expression; data are given in blocks of 3 CS. Figure 6 presents the data collapsed across trial blocks

P values are for Stress X Drug X Trial block 3-way ANOVA and *post hoc* least significant difference test: Trial block main effect: ++++ $p<0.0005$.